BACTERIOLOGICAL REVIEWS, June 1973, p. 197-214

Vol. 37, No. 2
Printed in U.S.A.

Use of Somatic Cell Hybrids for Analysis of the Differentiated State

FRANCES M. DAVIS AND EDWARD A. ADELBERG

Departments of Microbiology and Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

THEORETICAL CONSIDERATIONS

The techniques of somatic cell hybridization have provided a tool for investigating certain aspects of mammalian cell differentiation. The purposes of this review are (i) to illustrate the types of questions to which hybridization experiments have been addressed, (ii) to point out some of the limitations of this approach, and (iii) to summarize the findings which have been published to date.

The genetic totipotency of differentiated cells is now an accepted doctrine, according to which a differentiated cell arises during embryonic development by the "activation" of a specific set of genes. This activation is normally irreversible; the mechanism by which it occurs is unknown and remains a central problem of developmental biology and genetics.

Differentiation Versus Modulation

A sharp distinction must be made between the process of *differentiation*, during which a particular gene becomes expressed, and the process of *modulation*, whereby the activity of that gene is henceforth regulated in the differentiated cell.

Consider, for example, the enzyme tyrosine aminotransferase (TAT), which is uniquely expressed in cells of the liver. During development of the liver, the TAT structural gene[1] is activated, and experiments with somatic cell hybrids suggest that the developmental activation of the TAT gene reflects the disappearance of specific repressor activity (since TAT levels are greatly reduced in hepatoma/fibroblast hybrids [3, 44, 58]). In the process of differentiation, however, it appears that a different repressor gene is turned on; the product of this gene functions in the induction of TAT synthesis by glucocorticoid hormones, as demonstrated by Tomkins, Gelehrter, and their colleagues (64).

The distinction between the process of differentiation on the one hand, and the modulation of gene activity occurring in a differentiated cell on the other, has been blurred by the usage of the term "regulation" to apply to both processes. It is also confusing that the term "repressor" is used to refer to the molecules which may prevent the expression of certain genes in undifferentiated cells, as well as to the molecules which participate in gene modulation in differentiated cells. The *same* repressor cannot possibly be involved in both processes, since that which is involved in differentiation must, once present in the cell, remain active under all environmental conditions, while that which is involved in modulation must be able to respond to effector molecules such as inducers or corepressors. The former is part of the mechanism of differentiation; the latter is a product of differentiation.

[1] The term "structural gene" is used in the sense of Jacob et al. (37) to refer to genes that determine the structure of polypeptides, and whose expression may be controlled by the products of "regulator genes."

Two Types of Hybrids: Heterokaryons and Synkaryons

The general properties of somatic cell hybrids have been described in several recent reviews (1, 25, 35). For the purposes of this discussion, it is necessary here only to distinguish between the two types of hybrids which may be used in experiments on differentiation.

The first product of cell fusion is a multikaryon, containing one or more nuclei from each parental cell. If the parental nuclei are different, the fusion product is called a *heterokaryon*. If heterokaryons are incubated in a growth medium, a small fraction of them (rarely more than one in 10^4) may go on to become *synkaryons*, in which the parental nuclei are effectively merged. The process by which nuclear fusion takes place is not clear, but it may involve simultaneous mitoses and the formation of a single spindle, rather than the fusion of interphase nuclei (35).

The expression of differentiated traits (i.e., traits which are peculiar to one or a few of the many different kinds of cell in the body) has been studied in both heterokaryons and synkaryons. The former have the advantages that (i) all parental chromosomes may be assumed to be present, (ii) the exact ratio of parental genomes in the heterokaryon can be determined, and (iii) the high frequency of formation of heterokaryons makes it possible to measure gene expression by direct cytochemical analysis of fixed cells: it is not necessary to select for the outgrowth of rare hybrid clones on which to perform the assays. Heterokaryons have the disadvantages that (i) the multinucleated state persists for only a limited period of time, and this period may be shorter than that needed for interactions to be expressed; and (ii) some macromolecular activators or repressors may not pass the nuclear membrane boundaries.

Synkaryons, conversely, have the advantages that (i) chromosomal losses permit the rough mapping of repressor and activator genes, (ii) synkaryons can form clones which can grow indefinitely and be cultured to high cell densities for enzymological and other biochemical analyses, (iii) rare synkaryon clones can be detected by their outgrowth in selective medium, and (iv) the two parental genomes are

free to interact within a single nucleus. Synkaryons have the disadvantages that (i) their propensity for chromosome loss makes detailed karyotype analyses essential, (ii) the ratio of genomes contributed by the two parents must also be determined by karyotype analysis, and (iii) their low frequency of occurrence makes necessary a selection system for their detection and isolation, and this is not always possible.

In the following sections, we will discuss separately the results obtained in experiments with heterokaryons and those obtained in experiments with synkaryons.

The Differentiated State: Three Hypotheses

Although somatic cell hybridization experiments cannot explore the differentiation process directly, they can be used to test the predictions of three alternative, all-inclusive hypotheses concerning the ultimate state of genes in the differentiated cell.

Autonomous expression of the structural genes. Consider, for example, the structural gene for the protein albumin, which is expressed only in liver tissue. According to hypothesis (i), the activation of the albumin gene during differentiation of the liver cell represents a switch to a new, *autonomous steady state:* that is, regardless of the mechanism by which the switch occurs, the new steady state of albumin gene activity is maintained throughout the further lifetime of the cell and even through further gene replications, independent of any diffusible regulatory molecules. How such a permanent change in the state of a gene occurs is not known, but it is possible to devise some useful working hypotheses. For example, the primary event in differentiation might be the removal of a specific, chromosomally bound inhibitor, leading to a conformational change in the chromatin of the albumin gene region such that (i) the gene is expressed and (ii) the specific inhibitor cannot again be bound.

Continuous production of an activator. According to hypothesis (ii), the expression of the albumin gene results from the appearance during development of a specific, diffusible *activator* molecule. As pointed out by Davidson (16), however, in this hypothesis the primary event in differentiation is one step removed from the activation of the albumin gene: the gene for the activator molecule undergoes the switch to the new, autonomous steady state of expression, and only secondarily activates the albumin gene. There is a possible evolutionary advantage to such an indirect process: namely, a single activator might turn on an entire set, or battery, of tissue-specific genes, as hypothesized by Britten and Davidson (8). In fact, somatic cell hybrids can sometimes provide a test of the hypothesis of coordinated gene activation.

Discontinued production of a repressor. Hypothesis (iii) is simply the negative analogue of hypothesis (ii): a gene determining a specific diffusible *repressor* for albumin gene activity becomes inactive during development as the result of a switch to a new, autonomous steady state. In terms of the working hypothesis presented above, the removal of a specific, chromosomally bound activator might lead to a conformational change in the chromatin of the repressor gene region such that (i) the repressor gene is no longer expressed and (ii) the specific activator cannot again be bound.

The terms "activator" and "repressor" as used here are defined strictly operationally, and no particular mechanism is implied. Whereas, in *Escherichia coli*, in vitro systems of enzyme synthesis permit the direct demonstration of the negative (inhibitory) action of a repressor molecule (67) or the positive (stimulatory) effect of an activator molecule (68), the in vivo systems discussed below (i.e., mammalian cell hybrids) cannot distinguish between such direct actions and various indirect actions involving more than one step. Thus, what appears as an "activator" in its net effect on a gene, such as the structural gene for albumin, may not be a factor which participates directly and positively in the transcription or translation of that gene. Rather, it may be a molecule which inhibits the formation or action of a repressor of the albumin gene. If repressor formation were inhibited, the "activator" would, in fact, be a repressor of a repressor gene; if repressor action were inhibited, the "activator" would be an endogenous inducer, which neutralizes a repressor molecule by binding to it.

Similarly, a repressor may act directly to inhibit the transcription or translation of a given structural gene, or may act indirectly by interfering with the expression of an activator gene. Given such uncertainties concerning mechanisms, the statement that a given differentiated state is under "positive" or "negative" control has little meaning with respect to the details of the molecular mechanism.

Experimental Tests of the Hypotheses

The three hypotheses outlined above lead to different predictions concerning the outcome of hybridizations between a cell expressing a particular differentiated trait and one that does not. These predictions are presented below in their most simplified form, in order to illustrate

the utility of somatic cell hybrids for experimentally testing the three hypotheses. In the following section we will analyze the conditions under which these predictions are valid and will show how the predictions are altered as the conditions are changed.

Let us consider, for example, a hybrid between a liver cell, forming albumin as a differentiated product, and a fibroblast, which does not make albumin. Hypothesis (i) predicts the *continued expression* in the hybrid of the albumin genes contributed by the liver cell, and the continued inactivity of the albumin genes contributed by the fibroblast. Hypothesis (ii) predicts that the albumin genes of *both* parent cell types will be active: i.e., the albumin genes of the fibroblast will be turned on by the activator contributed by the liver cell. Finally, hypothesis (iii) predicts that the hybrid will express the albumin genes of *neither* parent: i.e., the activity of the albumin genes contributed by the liver cell will be extinguished by the action of the repressor genes contributed by the fibroblast.

Factors Which May Influence the Expression of Differentiated Traits in Hybrids

In the preceding section, we stated that the three general hypotheses of the differentiated state lead to different predictions concerning the outcome of hybridizations between differentiated and "undifferentiated" cells, provided that certain conditions are met. (We will use the term "undifferentiated" to refer to the parental genome which does not express the differentiated trait in question.) These conditions are as follows: (i) All of the parental genes involved in the normal expression of the trait in question must be present in the hybrid. (ii) If activators or repressors are involved, they must be present in excess and must bind equally well to the cognate receptors of both parental genomes. (iii) The hybrid cell must not possess unrelated properties which nonspecifically interfere with the expression of the trait in question.

We will now show how the predictions are influenced by changes in these conditions.

Qualitative gene content. Before it can be concluded that a particular gene is not being expressed in a given hybrid, it must be established that the gene is present. This is not a problem in heterokaryons; however, given the frequent occurrence of chromosomal abnormalities or loss in synkaryons, one cannot assume the presence in such hybrids of all genes originally contributed by the two parent cells.

There are two situations in which this problem arises: (i) a gene which was demonstrably active in one of the parent cells is no longer active in synkaryon, and (ii) a gene which was presumed to be contributed by one of the parent cells failed to be activated. The analysis of the latter situation is hopeless, unless the hybrid cell which is presumed to contain the gene can be tricked into revealing it—e.g., by induction, mutation, selection, or some combination of these. Without such information, the experimental results remain equivocal. In the former situation, however, if we assume the simplest model of repressor formation and action, the parental activity which has been extinguished in the hybrid should reappear if the repressing gene(s) are lost—e.g., by chromosome reduction. Since chromosome reduction is characteristic of somatic cell hybrids, there is a good chance that, by continuously propagating the hybrid over many cell generations, clones will be produced in which the extinguished property has reappeared. Such clones must be sought if the claim for extinction is to be valid.

It is true that more complicated models can be imagined, in which a gene activity once extinguished would not reappear when the hybrid lost the repressor genes contributed by the undifferentiated parent. For example, if a repressor served as its own activator, forming a positive feedback loop, both parental sets of repressor genes would be turned on in the hybrid, and both sets would have to be lost for reexpression to occur. There is no evidence for such a mechanism, however, whereas the simple model has been confirmed in several instances by the reappearance of extinguished traits in reduced hybrids. These cases will be described below.

Quantitative gene content. The relative genomic inputs of the two parent cells may influence the expression of a differentiated trait in a hybrid. Consider, for example, a repressor which is present at such low concentration in the undifferentiated parent that it cannot repress more than four copies of its target gene. In such a case, extinction would be observed in a 1:1 hybrid (one diploid genome from each parent) but not in a hybrid containing two genomes from the differentiated parent and one from the undifferentiated parent. In fact, competition for the repressor in the latter case could actually lead to some activation of the unexpressed genes contributed by the undifferentiated parent.

Analogous problems arise if a cell owes its differentiated state to the presence of very low levels of activator. First, let us consider the case where a "direct activator" is involved (i.e., one

which participates directly and positively in transcription or translation of the structural gene for a differentiated trait). In a 1:1 hybrid, some gene copies will surely be expressed; as the ratio of differentiated to undifferentiated genomic input is progressively either raised or lowered, the likelihood of cross-activation increases (as a result of excess activator when the ratio is raised, and of competition for activator when the ratio is lowered). When, however, an "indirect activator" is involved (i.e., one which acts by inhibiting the formation or action of a repressor), its presence in limiting quantities may actually lead to *extinction* in a hybrid, and the probability of this occurring increases as the ratio of undifferentiated to differentiated genome increases. Suppose, for example, that the structural gene for albumin is inactivated by a repressor in early embryonic cells, sufficient repressor being produced to repress four or more gene copies. Suppose further that the development of liver parenchymal tissue is characterized by the turning on of an inducer gene, whose product is present at a level just sufficient to neutralize that amount of repressor. In a liver cell/embryonic cell hybrid, the level of unneutralized repressor will then be sufficient to extinguish the formation of albumin completely. Similar stoichiometries may exist when the "activator" is an inhibitor of repressor formation, rather than of repressor action.

Specific binding of activators or repressors. Most investigators have deliberately chosen cells from two different animal species for their hybridization experiments, in order to be able to recognize the source of the expressed gene products. For example, rat and mouse albumin are immunochemically distinguishable; in rat/mouse hybrids that produce albumin, antiserum can be used to determine whether the albumin results from the activity of the rat structural gene, the mouse structural gene, or both (54).

The use of interspecific hybrids, however, may produce results that cannot be unequivocally interpreted. Thus the failure of an albumin-producing rat genome to activate an undifferentiated mouse genome in a hybrid could mean either that there is no activator present, or that an activator is present but has too low an affinity for the relevant mouse receptor. The same argument can be made, of course, concerning repressors, when extinction is not observed. The solution to this problem would be to use differentiated and non-differentiated cells from two different strains or cell lines from the same animal species, such that the gene product to be tested is *mutationally* different in the two parents.

Modifying factors. By "modifying factors," we mean all factors affecting the gene expression in question other than those which are directly related to the differentiation process. Periman (53), for example, suggested that the reduced excretion of immunoglobulin observed in a hybrid between an immunoglobulin-producing plasmacytoma cell and a non-producing fibroblast might be due, not to a repressor, but to the absence in the hybrid of an adequate secretion apparatus. In general, the expression of one differentiated trait may require the simultaneous expression of other differentiated traits, and these may not be coordinately controlled. It would be particularly important to know whether specialized cell components are required to translate the messenger ribonucleic acid (RNA) for any differentiated proteins in vivo, since, for example, the contribution of ribosomes by the two parental genomes may be far from equal (7, 24). The fact that heterologous ribosomes function in some cell-free protein synthesizing systems does not rule out an in vivo role for specialized ribosomes in some differentiated states.

Another possibility is that extinction represents the alteration of a differentiated gene product in the hybrid, rather than its absence. For example, Parkman et al. (52) showed that human thymocyte/mouse fibroblast hybrids produced an altered immunoglobulin, different from (although antigenically related to) that of the thymocyte parent. The alteration of differentiated gene products in hybrids could occur either during or subsequent to translation.

As an absolute minimum, the absence of a differentiated trait should not be attributed to specific repressor action until it has been shown that the hybrid in question is capable of expressing non-differentiated traits which were active in both parents: e.g., surface antigens (40, 62) or various metabolic enzymes (28, 45, 46, 66). Conversely, a positive result should not be attributed to a differentiation-specific activator, until selfed hybrids of the undifferentiated parent have been used to show that the new activity is not induced by the fusion process non-specifically.

Finally, the possibility of *masking* must be considered. For example, certain surface properties of macrophages are not found in macrophage/melanocyte heterokaryons, not because they are repressed, but because they are masked by cell-coat constituents produced by the melanocyte genome (33).

Interpretation of Experiments with Hybrids

Continued expression. Without further experiment, the simple observation of "continued expression" can be interpreted alternatively as evidence for (i) autonomous expression of the relevant structural genes (that is, the absence of any diffusible activators or repressors); (ii) (in interspecific hybrids) the highly specific binding of a repressor or an activator by the homologous parental genome, such that cross-activation or cross-repression cannot occur; (iii) (in heterokaryons) the failure of activators or repressors to pass through nuclear membranes; or (iv) (in synkaryons) the loss of chromosomes bearing activator or repressor genes or unexpressed structural genes.

Activation. Without further experiment, the simple observation of activation can be interpreted alternatively as evidence for (i) the contribution of an activator by the differentiated parent, (ii) the competition for limited quantities of a repressor produced by the genome of the undifferentiated parent, or (iii) (in synkaryons) the loss of repressor genes from the hybrid.

Extinction. Without further experiment, the simple observation of extinction can be interpreted alternatively as evidence for (i) the contribution of a repressor by the undifferentiated genome, (ii) the contribution by the differentiated genome of limiting quantities of an indirect activator (one which acts by inhibiting the formation or action of a repressor), (iii) the masking of the differentiated trait by a product of the undifferentiated genome, or (iv) (in synkaryons) the loss of structural genes for the differentiated trait.

Criteria for Distinguishing Between the Three Hypothetical States of the Differentiated Cell

With the above alternative interpretations in mind, we can now define the criteria for distinguishing between the three hypothetical states of the differentiated cell: autonomous expression, presence of activator, or absence of repressor activity.

Autonomous expression. For this state to be inferred, it must be shown that the two parental genomes do not influence each other's expression in hybrids: i.e., there is neither extinction of activity of the genes contributed by the differentiated parent, nor activation of the homologous genes contributed by the undifferentiated parent.

Consider, for example, the formation of acetylcholinesterase in synkaryon hybrids of mouse neuroblastoma/mouse fibroblast origin. Various levels of this enzyme have been observed in different hybrid clones (48, 49). In order to conclude that "autonomous expression" best describes the state of the acetylcholinesterase genes in these hybrids, the following criteria would have to be applied.

(i) The total amount of enzyme per cell should equal that of the neuroblastoma parent (assuming one neuroblastoma genome per hybrid cell).

(ii) The hybrid should be demonstrated to contain the entire genomes of both parents; otherwise, the absence of repression or activation might be due to the loss of chromosomal segments bearing repressor or activator loci. (This would require a detailed karyotype analysis, which at best could only show that no chromosomal segments were visibly absent.)

(iii) The enzyme should be shown to originate entirely from the neuroblastoma genome. (This would obviously require that the enzyme be mutationally different in the two parental cell lines, such that the two forms are distinguishable in extracts.)

Criteria (i) and (ii) above have never been fully satisfied, to our knowledge, in any experiments dealing with synkaryon hybrids between differentiated and undifferentiated cells. Many investigators have attempted to satisfy criterion (iii) by using parent cells from different animal species; this approach, however, suffers from the inability of the data to distinguish between the lack of repressor or activator, and the failure of such molecules to be bound by heterologous receptors (see above).

Production of activator. Production of activator is one of two possible explanations for the observation that, in a hybrid, the corresponding genes of the undifferentiated parental genome become expressed. The other possibility is that the undifferentiated parental genome produces repressor in a quantity which is insufficient to inhibit all of the target gene copies in the hybrid.

The latter hypothesis predicts that the rate of gene expression for the differentiated trait in question will never be greater in the hybrid than it is in a parental cell containing the equivalent dosage of genes for that trait. In other words, the gain of activity in the derepressed genome must be exactly balanced by the loss of activity in the newly repressed genome. The activator hypothesis, on the other hand, predicts a net gain in differentiated gene expression if one assumes an excess of activator to be produced.

Thus, if a net gain in differentiated gene expression can be demonstrated in the hybrid,

the repressor hypothesis becomes unlikely. If such net gain is not (or for technical reasons cannot be) demonstrated, a final choice between the two hypotheses cannot be made.

Absence of repressor activity. Given the following criteria, the conclusion that a differentiated trait owes its appearance to the loss of repressor activity is strongly supported

(i) The differentiated trait is partially or fully extinguished in the hybrid. (ii) If synkaryons are used, the differentiated trait reappears in subclones of the hybrid which have lost one or more chromosomes (or chromosomal segments) contributed by the undifferentiated parent. (iii) The trait (e.g., enzyme) which reappears in such subclones is identical to that which was extinguished in the original hybrid. (This cannot be taken for granted, given the common occurrence of multiple forms of enzymes [isozymes] in the same animal species, and the existence of different enzymes with overlapping substrate specificities. The latter may be particularly relevant to studies on transaminases [29].) (iv) The differentiated trait is not subject to masking by a product of the undifferentiated genome.

As we have shown earlier, however, these criteria do not distinguish between the absence of repressor and the presence of limited amounts of an inducer as the basis for the lack of repressor activity in the differentiated cell. Furthermore, even if the absence of repressor could be confirmed, our experimental system cannot distinguish between the lack of repressor as an *autonomous* steady state of the repressor gene, or as a consequence of the presence of an inhibitor of repressor gene expression. Each of these alternatives demands a different interpretation of the differentiation process: in one case, a repressor gene becomes inactive as new, autonomous steady state; in the second case, an inducer gene becomes active; and in the third case, a gene producing an inhibitor of repressor gene expression becomes active.

In all three cases, however, the net effect is the disappearance of *repressor activity* in the differentiated cell.

EXPERIMENTAL RESULTS

In the following sections, we will review the accounts which have been published to date of hybridizations between differentiated and undifferentiated cells. In each case, we will attempt to relate the results to the three basic hypotheses discussed above. All of the results to be described were obtained with synkaryons, unless otherwise noted.

Liver Properties

Tyrosine aminotransferase

Heterokaryons. Thompson and Gelehrter fused two liver-derived cell lines from the same inbred strains of rat: HTC (hepatoma) cells, which contain an inducible TAT, and BRL-62 cells, which do not contain detectable levels of this enzyme (63). In heterokaryons analyzed 18 to 24 h after fusion (equivalent to six to eight TAT half-lives), the expression of TAT and its inducibility were both extinguished. Both nuclei in the heterokaryon synthesized RNA, and amino acids were incorporated into protein; thus, the extinction of TAT synthesis was specific. Mixtures of lysates of the parental cell types showed no inhibition of TAT activity, so enzyme inhibition or degradation was probably not involved.

Synkaryons. The expression of TAT in synkaryon hybrids of rat hepatoma cells with mouse fibroblasts has been investigated by Thompson and his colleagues (3, 44) and by Schneider and Weiss (58). The hybrids resembled the fibroblast parent in containing low levels of a non-inducible enzyme having tyrosine transamination activity (3, 44, 58). Given the existence of transaminases with overlapping substrate specificities (29), it is quite possible that the fibroblast enzyme is not TAT. Thompson's laboratory found only the fibroblast type of enzyme in the hybrids (3, 44), while Weiss's laboratory interpreted heat-inactivation kinetics to suggest the presence in the hybrids of both parental forms (58).

The enzyme is not inhibited in mixtures of parental cell lysates (3). Actinomycin D did not stimulate TAT synthesis in the hybrids as it does in hepatoma cells (3). Chromosome loss in most of the hybrid clones was small, and probably random (3, 58). These results are compatible with the contribution by mouse fibroblasts of an active developmental repressor of inducible TAT synthesis. As we pointed out earlier, such a developmental repressor cannot be the same as the modulation repressor in liver cells, which controls TAT synthesis in response to inducers such as dexamethasone. The latter repressor is itself a differentiated trait.

Weiss and Chaplain (65) have described a hybrid between rat Fu-5 hepatoma cells and BRL-1, a TAT-negative cell line derived from rat liver. The TAT activity of Fu-5 was originally extinguished in this hybrid. After further passage, a clone appeared which had lost 30 to 40% of the chromosomes initially present, and which now exhibited a low level of tyrosine transamination activity which was increased

12-fold in the presence of dexamethasone. The induced levels, however, were at least 50-fold lower than in the inducer Fu-5 cells, so that it is not clear that the same enzyme is involved. Even if it is the same enzyme, it is not possible to say whether the new activity in the hybrid represents a reexpression of the Fu-5 TAT genes, or the activation of the BRL-1 TAT genes, since both parents were ultimately derived from TAT-positive tissue. For the same reason, a role in the normal process of differentiation cannot be inferred for the repressor contributed by BRL-1.

Albumin

Darlington, Bernhardt, and Ruddle (14) constructed a mouse hepatoma/human leukocyte hybrid which has retained very few human chromosomes. Cells of this hybrid synthesize both mouse and human albumin. These results cannot be interpreted in terms of differentiation mechanisms. The expression of the human albumin locus may reflect either the contribution of a mouse activator locus or the loss (through chromosome reduction) of a human repressor locus. The continued expression of the mouse albumin locus may reflect the absence of a cross-reacting repressor in diploid human cells or (again) its loss during chromosome reduction.

Peterson and Weiss (54) examined a series of rat hepatoma/mouse fibroblast hybrids with different ratios of parental genomes. In 1:1 hybrids, mouse albumin did not appear and rat albumin synthesis was partially extinguished, suggesting the contribution of repressor activity by the mouse fibroblast. Five hybrid clones were studied in which the ratio of rat-to-mouse genomes was 2:1. One of these produced mouse albumin as well as rat albumin. Such cross-activation could reflect either the contribution of rat activator, the competition for limited quantities of mouse repressor, or the loss of the mouse repressor during chromosome reduction.

Two of the 2:1 hybrids produced mouse albumin only, and two others produced neither rat nor mouse albumin. These results can be fitted to any of a large number of hypotheses invoking the loss of albumin structural genes, together with the presence or loss of hypothetical activator or repressor genes.

Aldolase B

Liver aldolase (aldolase B) is repressed in hybrids of rat Fu-5 hepatoma cells with mouse fibroblast strains 3T3 and LM, and also with rat liver-derived BRL-1 cells (5). When both parents possessed aldolase A, or aldolase A and aldolase C, the enzymes of both parental species appeared in the hybrids, showing that aldolase B repression was specific. In mixture experiments, mouse fibroblast and rat BRL-1 cell extracts were shown to be free of aldolase inhibitors. Aldolase B reappeared in an FU-5/BRL-1 hybrid clone which had undergone chromosome reduction (4). These results are subject to the same interpretational difficulties as the reexpression of TAT in hepatoma/BRL-1 hybrids discussed earlier.

Alcohol dehydrogenase

Liver-specific alcohol dehydrogenase (ADH), present in rat Fu-5 hepatoma cells, was extinguished in Fu-5/3T3 (mouse fibroblast) hybrids and also in Fu-5/BRL-1 hybrids (4). ADH activity reappeared in an Fu-5/BRL-1 clone which had undergone chromosome reduction. Once again, the results are subject to the same interpretational difficulties as the reexpression of TAT in hepatoma/BRL-1 hybrids discussed earlier.

Kidney Esterase

RAG, a cell line derived from mouse kidney proximal convoluted tubule, expresses the kidney-specific enzyme, esterase-2 (ES-2). ES-2 activity is extinguished in hybrids of RAG with mouse fibroblast L cells, and with human fibroblast WI-38 cells (42).

Human chromosomes are preferentially lost during the passage of the mouse/human synkaryons. Subclones isolated at different times during passage include ES-2-positive and ES-2-negative types; the ES-2-positive clones remain stably so during further passage, whereas the ES-2-negative clones continue to segregate both types.

These results are compatible with the contribution by the human cell parent of specific repressor activity for ES-2. Preliminary karyotype analyses suggested a correlation of ES-2 reappearance with the loss of human chromosome 10 (42), but further work has failed to confirm a correlation with the loss of any one specific human chromosome (F. Ruddle, unpublished data). The genetic basis for the repression thus appears to be complex.

Melanin

The extinction of pigment synthesis in melanoma cell hybrids has been reported. Davidson et al. (19) examined over 100 hybrid clones from a pigmented Syrian hamster melanoma/mouse fibroblast L cell cross; all were unpigmented

and lacked dopa oxidase activity, even though the L cells were derived from a mouse strain that was capable of synthesizing pigment (19). Davidson and his colleagues showed that the absence of pigment was probably not due to an inhibitor of dopa oxidase activity (21), the preferential fusion of rare, amelanotic melanoma cells (15), the loss of structural genes for melanin synthesis (20), or the non-specific inactivation of melanoma genes by the L-cell genome (20).

Silagi (59) found a similar repression to occur in an intraspecific cross of mouse melanoma X mouse fibroblast. The hybrid cells also formed unpigmented tumors, confirming in vivo the results obtained in vitro. However, in Silagi's report, the fusion of rare amelanotic cells was not excluded.

Both Fougère et al. (27) and Davidson (17) observed that 2:1 hybrids, resulting from the fusion of tetraploid melanoma cells with fibroblast L cells, could give rise to up to 50% pigmented colonies whose dopa oxidase varied cyclically in culture as did the dopa oxidase of the melanoma parent. Subcloning of pigmented clones again yielded a mixture of pigmented and unpigmented types, while the unpigmented phenotype remained stable (17). The unpigmented clones had an average of four to six fewer chromosomes than the pigmented clones.

These results are difficult to reconcile with the hypothesis of a repressor in the unpigmented cells, which would predict the genetic instability of unpigmented, rather than pigmented, hybrids. They are also difficult to reconcile with the hypothesis of an activator in the pigmented cells, which would predict at least some melanin or dopa oxidase production in 1:1 hybrids. The segregation of unpigmented cells from the pigmented hybrids may, however, reflect the loss of chromosomes bearing genes for melanin production, in which case the results would be compatible with extinction by a repressor in 1:1 hybrids. The appearance of melanin in 2:1 hybrids would then be due either to competition for limiting quantities of repressor, or to the increased contribution of indirect activator by the melanoma genomes. The failure of unpigmented hybrids to segregate pigmented cells through loss of repressor would be predicted if the chromosome bearing the hypothetical repressor gene also carried at least one structural gene essential for melanin synthesis.

Tadpole Versus Adult Frog Hemoglobin

Rosenberg (56) fused tadpole and adult frog nucleated erythrocytes, each of which synthesizes a different and distinguishable hemoglobin as a result of a switch in gene activities during development. She showed that both hemoglobins were synthesized in the tadpole/adult frog heterokaryons, but that tadpole hemoglobin synthesis was *reduced* twofold whereas adult frog hemoglobin synthesis was *increased* twofold.

These concomitant changes might reflect a switch in the heterokaryon gene activities similar to that which occurs during metamorphosis, in which case a model could be constructed involving the appearance during metamorphosis of tadpole hemoglobin repressor and an adult hemoglobin activator. Alternatively, the changes observed may indicate only that adult hemoglobin message is translated more efficiently by, and thus competes successfully for, the available ribosomes. One might distinguish between these possibilities by using cells from frogs with variant adult hemoglobins; in such an experiment it would be possible to tell whether the increased adult hemoglobin in the erythrocyte heterokaryons reflected a switching on of the "adult" genes contributed by the tadpole parent, or simply an enhanced rate of expression of the "adult" genes contributed by the frog parent.

Immunoglobulins

Several laboratories have reported the results of fusing immunoglobulin (Ig)-secreting cells with cells that do not synthesize Ig.

The earliest such report was that of Periman (53), who found that mouse plasmacytoma/mouse fibroblast L-cell hybrids secreted greatly reduced amounts of Ig relative to the amounts secreted by the plasmacytoma parent. He suggested that the observed extinction might be explained by an inefficient translation or secretion mechanism in the hybrid, rather than by an L-cell repressor.

Coffino et al. (11) hybridized Ig-producing mouse myeloma cells with mouse fibroblast 3T3 cells. Cell lysates, as well as supernatants of cell cultures, were analyzed for Ig. Even short radioactive pulses (given in case the immunoglobulins were rapidly degraded) failed to reveal Ig synthesis by the hybrids; as little as 1% of the level seen in the myeloma parent could have been detected by their procedure. The H2 antigens of both parents were present on the hybrid cell membranes, ruling out any general suppression of myeloma genome activity.

A second laboratory (43) similarly failed to detect Ig in hybrids; in this case, surface-bound Ig was assayed in hybrids of human lympho-

blasts (which possessed membrane-bound IgM) with mouse fibroblast A9 cells. In neither case, however, were subclones examined for the reappearance of Ig expression; thus, the presence of the Ig genes contributed by the Ig-positive parent was not directly demonstrated. The results could thus be due either to the absence of the Ig genes or to the presence of a repressor activity. It is also possible that the hybrids were producing altered Ig's which would escape detection by the methods used; this possibility is raised by the following report.

Parkman et al. (52) found that up to 60% of their human thymocyte/mouse fibroblast hybrid clones were positive for membrane-bound Ig when first tested, but became negative after further cultivation. The Ig of the hybrids was partially identical (in immunodiffusion) to whole gamma globulin and to Fab fragments, but was not identical (on acrylamide gels) to whole gamma globulin, Fab, Fc, or light chains.

These results indicate a much more complex interaction of genomes in the hybrids than simple extinction or activation. The altered Ig detected could reflect activity by the fibroblast genome at any of several levels, including translation and post-translational modification. The final disappearance of Ig from the hybrid membranes, and its failure to be detected at all in the studies reported earlier, could also reflect a masking of surface Ig by cell coat constituents provided by the fibroblast genome.

One other positive report has appeared. Mohit and Fan (50) fused Ig-positive mouse myeloma cells, which produce both Ig and free κ light chains, with mouse lymphoma cells which produce neither. The hybrids had the membrane antigens of both parents, and secreted κ light chains; no heavy chains were detected. These results, however, do not permit any useful interpretations of control mechanisms since: (i) it is not known which parental genome in the hybrids was producing the κ chains; (ii) the observed activity could reflect the loss of repressor genes from the hybrid; (iii) the absence of other Ig polypeptides could similarly reflect the loss of the corresponding structural genes; (iv) the Ig-negative parent was presumably a dedifferentiated rather than an undifferentiated cell; and (v) the production of free κ chains by the myeloma parent is an abnormal phenotype, and unregulated κ chain production might be due to an inability of the myeloma gene to be repressed. Numerous other speculations are also possible.

Macrophage Surface Properties

The extinction of macrophage traits in heterokaryons formed by the fusion of rat macrophages and mouse melanocytes was reported by Gordon and Cohn (30, 33). About 5 days after fusion, the heterokaryons lost differentiated properties characteristic of the macrophage: ability to phagocytize opsonized erythrocytes, high levels of a Mg^{2+}- or Ca^{2+}-dependent adenosine triphosphatase, uptake of dextran sulfate, presence of refractile lipid droplets, and general macrophage morphology (30). The loss of the macrophage properties was retarded by increasing the ratio of macrophage: melanocyte nuclei in the heterokaryons, and prevented by irradiation of the melanocyte nucleus before fusion or by inhibition of RNA and protein synthesis at the time of fusion (33).

The extinction of the macrophage surface properties, however, was not due to repression at the genetic level but rather to masking at the cell surface level, since phagocytosis could be recovered by mild trypsinization (33). This result emphasizes the importance of ruling out the masking of a differentiated gene product, before concluding that the gene has not been expressed. In the present example, the best interpretation would seem to be "continued gene expression" of both parental genomes, since neither repressors nor activators need be invoked to explain the results.

Neuronal Traits

Heterokaryons. DiZerega and Morrow (22) fused chicken perikarya neurons with HeLa (cultured human cervical carcinoma) cells, and studiedthe stimulation by nerve growth factor of the extension of long processes from the heterokaryons. They found that the processes so formed were shorter and less well-developed than the axons of normal neurons. These results might be interpreted as either dilution or partial extinction of the differentiated trait; however, the specificity of the effect was not checked by the use of control neuron/neuron fusions.

Synkaryons. Many neuronal properties continue to be expressed in mouse neuroblastoma/mouse fibroblast L cell synkaryons (47–49). The neuronal traits examined included electrical response, acetylcholinesterase activity and its regulation in the cell culture cycle, response to acetylcholine, and the presence of neurites and neurofibrillar protein. All of these traits can be expressed in some hybrids, identified as such by marker chromosomes and by the presence of parental isozymes of glucose phosphate isomerase and phosphoglucomutase (48). Other hybrids express only some of these traits. The combinations observed were not random, and on the assumption that these combinations represented permissible patterns of expression,

Minna et al. inferred from them a possible sequence of steps in neuron maturation (48). Hybrids with fewer neuronal traits, however, generally have fewer chromosomes (48), so that an equally plausible explanation of the observed combinations is that they reflect patterns of loss of structural genes from the hybrids, rather than patterns of activity of developmental activators or repressors.

Some hybrids exhibited no neuronal traits (48). Assuming the presence of the relevant structural genes, this suggests extinction of neuronal gene expression by the fibroblast L-cell genome. Some hybrids possessed higher levels of neuronal traits than did the neuroblastoma parent (48, 49); this result could be explained by a variety of hypotheses, including cross-activation of the fibroblast genome, modulation of neuroblastoma genes, and the loss of repressor genes from the hybrid.

Human neuroblastoma/mouse fibroblast hybrids have been studied by McMorris and Ruddle (unpublished data). Only one or two human chromosomes were retained in some hybrids which had high acetylcholinesterase levels, characteristic of the neuroblastoma (but not the fibroblast) parent. The human chromosomes retained were not specific; thus, either the human acetylcholinesterase gene had been translocated to a mouse chromosome, or the mouse (fibroblast) acetylcholinesterase genes were activated. The latter seems more likely, since no translocations were visible in the hybrid karyotypes. If activation has occurred in this system, it could reflect any of the processes we have described earlier: activity of a human activator, titration of a mouse repressor, or loss of mouse repressor genes.

Glial Cell Traits

The expression of the following glial cell traits has been studied in hybrids: morphological characteristics (57), S-100 protein, a neural-specific acidic protein (2), the inducibility of lactic dehydrogenase (LDH) by epinephrine (18), and the high baseline level of glycerol-3-phosphate dehydrogenase (GPDH) as well as its inducibility by hydrocortisone (18). Glial morphology, S-100 protein, LDH inducibility, and GPDH inducibility are all extinguished in rat glial cell/mouse fibroblast synkaryons (2, 18, 57). The presence of repressor(s) for these traits in fibroblasts is suggested by these results, but final proof will require demonstration of the reappearance of the traits in segregants; otherwise, structural gene losses may be responsible. The hybrids contain small amounts of a protein which cross-reacts immunochemically with antibodies against the S-100 protein (2). The cross-reacting material could either be S-100 protein which has been altered in the hybrid, or the product of a totally different gene. It appears not to be native S-100 protein since, when complexed with anti-S-100 antibody, it fixes less complement than does the native material.

In contrast to the above results, the baseline level of GPDH approximated the high level of the glial parent in synkaryons receiving two glial genomes and one fibroblast genome, while exhibiting the low-level characteristic of the fibroblast parent in 1:1 synkaryons (18). GPDH was not inducible in the 2:1 hybrids; the baseline level and inducibility of GPDH are thus not coordinately controlled. The extinction of the high baseline level in 1:1 hybrids and its reappearance in 2:1 hybrids is compatible with (i) the presence in fibroblasts of limiting quantities of a specific repressor which plays no role in induction, or (ii) the presence in glial cells of limiting quantities of indirect activator. The presence of direct activator in the glial cells is less likely, since this hypothesis would predict intermediate baseline activity in 1:1 hybrids.

Cessation of Nucleic Acid Synthesis

Deoxyribonucleic acid (DNA) replication does not occur in a number of differentiated vertebrate tissues, and both DNA and RNA synthesis are inactive in some vertebrate erythrocytes which retain their nuclei. These phenomena are thus exceptions to the general rule that differentiation results from the activation of specific sets of genes.

DNA synthesis in mammalian macrophages, lymphocytes, and neurons. In a number of mammalian tissues, the differentiation process includes the cessation of DNA synthesis. The basis for this change has been examined in heterokaryons of mouse peritoneal macrophages with mouse melanocytes (30, 31), rabbit macrophages and rat lymphocytes with actively growing human HeLa cells (35), and mouse neurons with green monkey fibroblasts (38). In all four cases, the inactive nuclei resumed DNA synthesis in the heterokaryons, as shown by autoradiography of incorporated radioactive thymidine. That the results were not due simply to cell fusion was shown (in two of the cases) by fusing rabbit macrophages with each other and with rat lymphocytes; no DNA synthesis was detectable in these heterokaryons.

The above results are compatible with the absence in the differentiated nuclei of DNA polymerase or a factor required for its activity, and the transfer of polymerase or activating factor from the undifferentiated nuclei (HeLa or

fibroblast). The data presented do not rule out, however, the presence of limited quantities of repressor in the differentiated nuclei, for which the undifferentiated nuclei compete.

DNA and RNA synthesis in chicken erythrocytes. The inactivity of the avian erythrocyte nucleus is a differentiated property; during development, these nuclei cease all gene replication and transcription. Do these cells differentiate by ceasing to produce polymerases or activators of polymerases, or by commencing to produce specific repressors?

This question has been under experimental attack since 1966, particularly in the laboratories of Henry Harris and Nils Ringertz (6, 9, 10, 23, 35, 36, 55). The major conclusions from these studies can be summarized as follows.

(i) During the fusion of human HeLa cells with chicken erythrocytes by inactivated Sendai virus, the erythrocytes are hemolyzed and contribute no detectable cytoplasm to the resulting heterokaryons (35).

(ii) The following parameters of the erythrocyte nucleus increase over the ensuing 48 h: volume, dry mass, susceptibility of DNA to thermal denaturation, reactivity to Feulgen stain, and the binding by DNA of acridine orange and ethidium bromide (6, 35).

(iii) DNA synthesis commences at about 48 h (35).

(iv) RNA synthesis (transcription) begins within a few hours after fusion; nucleoli are present at the end of 2 to 3 days, after which chicken-specific (erythrocyte determined) membrane antigens appear (35).

(v) HeLa (human) nucleoplasmic and nucleolar antigens are specifically concentrated in the chicken erythrocyte nucleus as it undergoes activation (23, 55).

(vi) The rate of reactivation of erythrocyte nuclei decreases as the ratio of erythrocyte to HeLa nuclei increases in the heterokaryon (23).

(vii) A histochemical assay for RNA polymerase was developed, in which cells fixed to glass slides are provided with labeled nucleotide-triphosphates and the amount of RNA synthesized is measured by autoradiography (51). α-Amanitin-sensitive polymerase, known to produce heterogeneous nuclear RNA, was shown to be localized in the nucleoplasm. α-Amanitin-resistant activity was shown to be localized in the nucleoli; the nucleolar activity is more sensitive to actinomycin D than is nucleoplasmic activity.

Using this procedure, Carlsson et al. measured the activity of RNA polymerases in the erythrocyte nuclei of rat epithelial cell/chicken erythrocyte heterokaryons (9). α-Amanitin-sensitive (nucleoplasmic) polymerase was present by 24 h and increased steadily during the 96-h observation period; actinomycin-sensitive (nucleolar) polymerase appeared at about 48 to 72 h and increased thereafter. The rate of polymerase increase was proportional to the ratio of epithelial to erythrocyte nuclei in the heterokaryons.

The above results are compatible with the hypothesis that the erythrocyte nucleus lacks either RNA polymerase or a factor required for its activity, and that erythrocyte nuclei in heterokaryons receive polymerase, or activators, or both from the active nucleus. To rule out the alternative hypothesis (i.e., that the active nucleus competes for a limited amount of polymerase repressor produced by the erythrocyte nucleus), it would be necessary to show a net increase in the combined RNA polymerase activities of the two types of nucleus in the heterokaryon; such data have not to our knowledge been reported.

Myosin

Carlsson et al. (10) fused rat myoblasts with chicken erythrocytes and observed the reactivation of DNA, RNA, and protein synthesis by the erythrocyte nuclei. Rat myotubes containing reactivated chicken erythrocyte nuclei were reported sometimes to contain fibrils which stained with antibodies specific for chicken myosin. Thus, the gene for a muscle-tissue-specific product was sometimes activated in the erythrocyte nuclei. Such cross-activation reflects either the production of a myosin gene activator by the myoblast nuclei, or competition for limiting quantities of erythrocyte-produced specific repressor.

Ringertz and his colleagues (unpublished data) have observed 18 mononucleated cells among the above heterokaryons. These unusual cells produced chick myosin, were present in groups of two to four cells each, and probably represented clones of rat/chick synkaryons in which the gene for myosin had been turned on. These cells were not shown to produce rat myosin although hybrid myotubes formed by spontaneous fusion of rat and chick myoblasts were found to produce both rat and chick myosin; therefore, coexpression can occur if both of the input cell types are programmed to undergo myogenic differentiaton. These cells appeared to have at least as much DNA per nucleus as a rat myoblast. They did not form isolatable clones, however, so that further analysis (e.g., karyotyping) was not possible. Therefore, it cannot be said whether the activation of

the chick myosin genes in these hybrids reflected the contribution of an activator by the rat myoblast, or the loss or titration of chicken repressor.

Complement C4

Guinea pig peritoneal exudate (PE) cells synthesize the C4 component of complement. Colten and Parkman (13) made synkaryon hybrids between PE cells from guinea pigs homozygous for C4 deficiency and human HeLa cells which make no C4. The hybrids produced hemolytically active, human C4.

Karotype analysis revealed that very few guinea pig chromosomes and reduced numbers of HeLa chromosomes were retained in the hybrids; thus, the results did not distinguish between presence of a guinea pig activator or loss of a human repressor gene. However, Colten went on to show that C4-deficient guinea pig PE cells excrete into the medium a factor which promotes the synthesis of human C4 by HeLa cells (12). Total protein synthesis is not affected by the factor, and actinomycin D, puromycin, and cycloheximide inhibit both the production of the factor and the HeLa cell response to the factor.

Thus, guinea pig PE cells produce and excrete a protein "activator" of C4 synthesis which is missing in HeLa cells. Whether the excreted protein is a direct activator (as defined earlier) or an inhibitor of a repressor remains to be shown.

Pituitary Growth Hormone

Growth hormone is produced by a line of rat pituitary cells, but is extinguished in hybrids between rat pituitary cells and mouse fibroblast L cells (60, 61). A repressor in L cells is suggested, but the presence in the hybrids of the rat structural gene for growth hormone was not demonstrated (e.g., by the criteria of the reappearance of hormone in segregants).

Teratoma Differentiation

Finch and Ephrussi (26) and Jami et al. (39) hybridized L cells with cloned cells of a multipotential mouse teratoma which do not differentiate in vitro but, when reimplanted, give rise to tumors containing such differentiated tissues as neuroepithelium, cartilage, mesenchyme, keratin pearls, pigmented cells, and bone. Implantation of hybrid clones yielded tumors with no differentiated elements. Thus, L cells contain repressors for these differentiated gene expressions. Another possibility, which has not been excluded, is that genes essential for the expression of the differentiated traits had been lost in the hybrids. This explanation is rendered more plausible by the observation that, in one study, cells of the derived tumors had lost six to seven of the original hybrid chromosomes (39).

DISCUSSION

A cell which expresses a particular structural gene as a differentiated property may differ from one that does not in one of three ways: the two gene states may be autonomous, requiring neither diffusible activators nor diffusible repressors for their maintenance; the differentiated cell may continuously synthesize a diffusible activator of the structural gene; or the undifferentiated cell may continuously synthesize a diffusible repressor of the structural gene.

By analyzing the state of gene expression in somatic hybrids between two such cells, it is sometimes possible to distinguish among the three alternative mechanisms. This follows from the fact that each mechanism predicts a unique state of gene expression in the hybrid, provided that certain experimental conditions are satisfied. Thus, the hypothesis of autonomous expression predicts that the genes in the hybrid will retain the different states of expression which they exhibited in the parental cells; the hypothesis of a diffusible activator predicts that both sets of parental genes for the differentiated trait will be active; and the hypothesis of a diffusible repressor predicts the partial or complete extinction of the gene activity contributed by the differentiated parent.

In the first section of this review, we discussed a number of experimental shortcomings which can render such results ambiguous. The major problems which have been discussed are the following.

(i) In synkaryons, in contrast to heterokaryons, it is extremely difficult to establish that all the relevant genes are present. Thus, if a gene presumed to have been contributed by an undifferentiated parent fails to be activated in a hybrid, the result may reflect the loss of the structural gene or the activator gene, rather than the nonexistence of the latter. Only rigorous karyotype analysis can minimize this objection. Even with heterokaryons, the use of aneuploid cells as parents prevents one from assuming that an unexpressed gene has actually been contributed to the hybrid, since it may already have been lost from the parent cell.

(ii) The interpretation of cross-activation requires the precise quantification of the rate of gene expression in the parents and in the hybrid, and this is often difficult if not impossi-

ble to achieve. Without such quantitative data, however, it is not possible to distinguish between the action of an activator and competition for a limited quantity of repressor.

(iii) When hybrids are made between cells derived from different animal species, the absence of cross-activation or repression may reflect differences in binding affinities, rather than the absence of activators or repressors.

(iv) A differentiated trait may be cryptic, or masked, in the hybrid, even though the hybrid cells is transcribing and translating the gene in question.

(v) The parental nuclei may indiscriminately and non-specifically suppress each other's functions in the hybrids.

In the section on Experimental Results, we show that the above problems have been frequently encountered— although not always acknowledged— by the investigators. A few clear-cut cases of extinction have supported the unequivocal conclusion that the undifferentiated cell contains repressor activity which the differentiated cell lacks: TAT in HTC/BRL-62 heterokaryons (63); aldolase B and ADH in hepatoma/BRL-1 synkaryons (4); and esterase-2 in RAG/WI-38 synkaryons (42). These experiments, however, cannot tell us the reason for the lack of repressor activity in the differentiated cell. As we discussed in the section on Theoretical Considerations, such lack could reflect either the inactivity of the repressor gene as an autonomous steady state, the presence of an inhibitor of repressor gene expression, or the presence of an inhibitor of repressor gene activity. The last two alternatives would be examples of indirect activator production by differentiated cells.

One case of cross-activation has led to the actual isolation of an activator: complement C4 in guinea pig peritoneal exudate cell/HeLa cell synkaryons (12, 13). All other experiments have suffered from so many of the problems listed above that no unambiguous conclusions concerning mechanism can be drawn from them.

The immediate question which one attempts to answer by hybridizing differentiated with undifferentiated cells is: What is the basis of the difference between their two states of expression of the same structural gene? Behind this question, however, lies another of much greater general interest: How did this difference arise? In other words, what can experiments with hybrid cells tell us about the *process* of differentiation?

The answer to the latter question depends on the extent to which the two cell types which have been hybridized resemble the normal tissues of the embryo as they exist before and after the particular differentiated trait has appeared. Consider, for example, the cell which expresses the differentiated trait. In only a few instances, involving blood cells such as macrophages and erythrocytes, have normal cells been used in hybridization experiments. In all other experiments the "differentiated" cell has been a continuously growing tumor cell line, abnormal in many of its functions and usually aneuploid. The mechanism responsible in such cells for the maintenance of the differentiated trait in question may very well be secondarily acquired during tumorigenesis or adaptation to tissue culture, and may not reflect the normal process of differentiation.

The same ambiguity applies to the cell which, as the starting material for hybridization experiments, does not express the differentiated trait in question. In the great majority of experiments this cell has been a fibroblast, usually one that is adapted to continuous culture and is aneuploid. In a few cases the "undifferentiated" partner has been a euploid fibroblast of limited lifespan, and in another few it has been a tumor-derived line such as HeLa.

Each of these types poses a different problem of interpretation with regard to its significance for the normal process of differentiation. Thus, the genome of a continuously cultivated cell or of a tumor cell may have undergone such changes that it will behave quite differently in a hybrid from the way in which the genome of an undifferentiated, early embryonic cell would behave. Even the euploid fibroblast cannot be considered to be a reliable model of what we might call a "pre-differentiated" embryonic cell. The fibroblasts which most investigators use in experiments are themselves differentiated, e.g., with respect to collagen production. By the time a cell has differentiated to produce substance A, does it still retain the *capacity* to produce substance B which is characteristic of a different tissue? Perhaps it does not. Perhaps a fully differentiated cell acquires, as a final "fixation" step in its development, a set of secondary repressors which destroy its capacity to respond further to any other developmental signal. The repressors whose existence has been inferred from hybridization experiments with fibroblasts may in fact belong to this category, and may not be responsible for the absence of differentiated traits in early embryonic cells.

Finally, several investigators have used what appear to be dedifferentiated cells—e.g., tumor cells which retain some, but not all, of the differentiated properties of the parent tissue. It is by no means clear that dedifferentiation is

simply the reversal of differentiation. Rather, it may result from the appearance of new repressors or the loss of cell components which are necessary for the appearance of the property in question. If such a cell is hybridized with one which expresses the tissue-specific property, the results may be telling us about the differences between the differentiated and dedifferentiated states, rather than between the differentiated and undifferentiated states.

A number of such experiments have been reported. For example, a mouse fibroblast that does not produce hyaluronic acid and collagen has been hybridized with a fibroblast that does (34); a rat liver-derived cell that does not produce hepatic tyrosine aminotransferase has been hybridized with a rat hepatoma cell that does (63, 65); and a Chinese hamster ovary-derived cell that does not produce three particular ovarian esterases has been hybridized with a human fibroblast that also does not (40). In each case, the results are probably telling us more about the dedifferentiated than the undifferentiated state. In the last case, for example, the human fibroblast genome was observed to activate the hamster ovary genome to produce hamster ovarian esterases: the activator so revealed, however, seems unlikely to be the one whose absence would be responsible for the lack of esterases in developmentally competent, undifferentiated hamster embryo cells, and whose appearance during hamster ovary development would be responsible for esterase production.

If we are trying to answer the question which we stated earlier—that is, "What is the nature of the change which occurs when an undifferentiated cell becomes a differentiated cell?"—then we must obviously use only developmentally competent undifferentiated cells. But it is difficult, if not impossible, to recognize this state, short of using the fertilized egg itself. The closest one might come, perhaps, would be to use a cell from an early embryo or a teratoma cell, which is demonstrably capable of giving rise to many differentiated tissues when implanted in a host (39). The differentiations undergone by teratoma cells are abnormal in many respects, however, so that even this type of cell is not a perfect model of the developmentally competent undifferentiated state.

Interestingly, hybridizations of teratoma cells have been carried out, but for the opposite purpose of determining whether an undifferentiated fibroblast (a mouse L cell) would extinguish the ability of the teratoma cell to differentiate (26, 39). It did—which leaves us with the question of whether the L cell retains the capacity for further differentiation or has undergone a terminal fixation event. If the former is true, then its repressors are those whose loss is involved in the differentiations observed in teratomas; if it is "fixed," its repressors have been secondarily acquired and tell us nothing about the normal differentiation process.

A secondary question to which experiments with somatic cell hybrids may be addressed concerns the coordinate expression of differentiated traits: Does a single repressor or activator coordinately control the expression of two or more traits characteristic of a particular differentiated cell? If so, two related differentiated traits which have been extinguished in a hybrid should reappear simultaneously whenever the hybrid loses the putative repressor gene; conversely, related traits which have been cross-activated in a hybrid should disappear simultaneously whenever the hybrid loses the putative activator gene.

Positive evidence for coordinate control would be very difficult to obtain, given the present state of the experimental system, since hybrids tend to lose whole chromosomes (or major segments of chromosomes). Thus, the simultaneous reappearance or disappearance of related differentiated traits in a hybrid could reflect the simultaneous loss of two linked genes, rather than the loss of a single gene. Negative evidence, on the other hand, would be entirely conclusive: i.e., if the loss of a chromosome caused the reappearance or disappearance of one, but not the other, of two related traits. Such negative evidence has been reported above in one instance: the baseline level and inducibility of GPDH are not coordinately controlled in glial cell differentiation (18).

CONCLUSIONS

In the preceding sections we have discussed a number of serious difficulties in the interpretation of experiments with hybrids between differentated and undifferentiated cells. Many of these difficulties could be minimized, if not overcome, by the following experimental devices.

(i) The undifferentiated cell parent should be in the developmentally competent state, if the results are to have significance for the process of differentiation. Teratoma cells may meet this criterion; it might be even better to use cells taken directly from early embryos.

(ii) The problem of assuring the presence in hybrids of all relevant genes can best be met by using euploid parental cells and carrying out the analyses on heterokaryons whenever the appropriate cytological techniques permit. If synkary-

ons must be used, the presence therein of all parental chromosomes must be determined by detailed karyotype analysis, and even this procedure cannot assure the presence of all parental genes.

(iii) When cross-activation is observed, the rate of gene expression (transcription and/or translation) should be measured in the parent cells and hybrid; if the hybrid activity significantly exceeds that of the equivalent parental genomes, the possibility of competition for a repressor can be minimized.

(iv) Cells from different animal species should not be used, since uncertainties concerning binding affinities render many types of results ambiguous. Instead, the differentiated and undifferentiated cells should be taken from two different strains of the same animal species, in which the gene product to be tested is mutationally different and distinguishable.

(v) When a differentiated trait fails to appear in a hybrid, its presence in cryptic or masked form should be sought. For example, an enzyme characteristic of the differentiated parent may be produced in the hybrid but be inhibited by a product of the undifferentiated genome; this possibility should always be tested by assaying the enzyme in mixtures of extracts from the two parental cell types. Or, a differentiated surface property may be masked by a cell coat component produced by the undifferentiated genome, and may be revealed by treatment of the hybrid cells with hydrolytic enzymes.

(vi) Non-specific cross-suppression should be ruled out by demonstrating the ability of the hybrid to express a variety of non-differentiated traits of both parents (e.g., isozymes and surface antigens).

These recommended procedures are admittedly easier to prescribe than to carry out, but to ignore them is to introduce a large measure of ambiguity in the results. Even if all of them are followed, the interpretations of the results will contain that irreducible minimum of uncertainty that characterizes all experiments with living cells. Ultimately, the mechanisms of differentiation which we infer from experiments on somatic cell hybrids will have to be confirmed and elucidated in cell-free systems, as has been accomplished for the regulation of certain genes in *E. coli* (67, 68). This feat was made possible by the development of techniques for the isolation of DNA highly enriched for one particular set of genes, including a structural gene and adjacent binding sites for regulatory molecules. Only when techniques have been developed for the isolation of genes of higher organisms, which are differentially expressed during development, will the molecular mechanisms of differentiation be completely elucidated.

ACKNOWLEDGMENTS

We are grateful to R. L. Davidson, T. D. Gelehrter, and R. S. Krooth for their critical review of the manuscript and to F. H. Ruddle for making available data which have not yet been published.

LITERATURE CITED

1. Barski, G. 1970. Cell association and somatic cell hybridization. Int. Rev. Exp. Pathol. **9**:151-190.
2. Benda, P., and R. L. Davidson. 1971. Regulation of specific functions of glial cells in somatic hybrids. I. Control of S-100 protein. J. Cell. Physiol. **78**:209-216.
3. Benedict, W. F., D. W. Nebert, and E. B. Thompson. 1972. Expression of aryl hydrocarbon hydroxylase induction and suppression of tyrosine aminotransferase induction in somatic-cell hybrids. Proc. Nat. Acad. Sci. U.S.A. **69**:2179-2183.
4. Bertolotti, R., and M. C. Weiss. 1972. Expression of differentiated functions in hepatoma cell hybrids. VI. Extinction and re-expression of liver alcohol dehydrogenase. Biochimie **54**:195-210.
5. Bertolotti, R., and M. C. Weiss. 1972. Expression of differentiated functions in hepatoma cell hybrids. II. Aldolase. J. Cell. Physiol. **79**:211-223.
6. Bolund, L., N. R. Ringertz, and H. Harris. 1969. Changes in the cytochemical properties of erythrocyte nuclei reactivated by cell fusion. J. Cell Sci. **4**:71-87.
7. Bramwell, M. E., and S. D. Handmaker. 1971. Ribosomal RNA synthesis in human-mouse hybrid cells. Biochim. Biophys. Acta **232**:580-583.
8. Britten, R. J., and E. H. Davidson. 1969. Gene regulation for higher cells: a theory. Science **165**:349-357.
9. Carlsson, S., G. P. M. Moore, and N. R. Ringertz. 1973. Nucleo-cytoplasmic protein migration during the activation of chick erythrocyte nuclei in heterokaryons. Exp. Cell Res. **76**:234-241.
10. Carlsson, S. A., N. R. Ringertz, and R. Savage. 1971. Gene expression in hybrid myoblasts and myotubes produced by fusing rat myoblasts with various differentiated chick cells. Exp. Cell Res. **67**:243-244.
11. Coffino, P., B. Knowles, S. G. Nathenson, and M. D. Scharff. 1971. Suppression of immunoglobulin synthesis by cellular hybridization. Nature N. Biol. (London) **231**:87-90.
12. Colten, H. R. 1972. *In vitro* synthesis of a regulator of mammalian gene expression. Proc. Nat. Acad. Sci. U.S.A. **69**:2233-2236.
13. Colten, H. R., and R. Parkman. 1972. Biosynthesis of C4 (fourth component of complement) by hybrids of C4-deficient guinea pig cells and HeLa cells. Science **176**:1029-1036.

14. Darlington, G. J., H. P. Bernhard, and F. H. Ruddle. 1973. Activation of a human differentiated phenotype (albumin synthesis) in mouse hepatoma X human leukocyte somatic cell hybrids. Abstracts 24th Annual Meeting of the Tissue Culture Association. In Vitro, in press.
15. Davidson, R. 1969. Regulation of melanin synthesis in mammalian cells, as studied by somatic hybridization. III. A method of increasing the frequency of cell fusion. Exp. Cell Res. **55**:424–426.
16. Davidson, R. L. 1971. Regulation of gene expression in somatic cell hybrids: a review. In Vitro **6**:411–426.
17. Davidson, R. L. 1972. Regulation of melanin synthesis in mammalian cells: effect of gene dosage on the expression of differentiation. Proc. Nat. Acad. Sci. U.S.A. **69**:951–955.
18. Davidson, R. L., and P. Benda. 1970. Regulation of specific functions of glial cells in somatic hybrids. II. Control of inducibility of glycerol-3-phosphate dehydrogenase. Proc. Nat. Acad. Sci. U.S.A. **67**:1870–1877.
19. Davidson, R. L., B. Ephrussi, and K. Yamamoto. 1966. Regulation of pigment synthesis in mammalian cells, as studied by somatic hybridization. Proc. Nat. Acad. Sci. U.S.A. **56**:1437–1440.
20. Davidson, R., B. Ephrussi, and K. Yamamoto. 1968. Regulation of melanin synthesis in mammalian cells, as studied by somatic hybridization. I. Evidence for negative control. J. Cell. Physiol. **72**:115–128.
21. Davidson, R., and K. Yamamoto. 1968. Regulation of melanin synthesis in mammalian cells, as studied by somatic hybridization. II. The level of regulation of 3,4-dihydroxyphenylalanine oxidase. Proc. Nat. Acad. Sci. U.S.A. **60**:894–901.
22. DiZerega, G., and J. Morrow. 1970. The effect of nerve growth factor on dispersed neuronal-HeLa heterokaryons. Exp. Neurol. **28**:206–212.
23. Ege, T., S. A. Carlsson, and N. R. Ringertz. 1971. Immune microfluorimetric analysis of the distribution of species specific nuclear antigens in HeLa-chick erythrocyte heterokaryons. Exp. Cell Res. **69**:472–477.
24. Eliceiri, G. L. 1972. The ribosomal RNA of hamster-mouse hybrid cells. J. Cell Biol. **53**:177–184.
25. Ephrussi, B. 1972. Hybridization of somatic cells. Princeton University Press.
26. Finch, B. W., and B. Ephrussi. 1967. Retention of multiple developmental potentialities by cells of a mouse testicular teratocarcinoma during prolonged culture *in vitro* and their extinction upon hybridization with cells of permanent lines. Proc. Nat. Acad. Sci. U.S.A. **57**:615–621.
27. Fougère, C., F. Ruiz, and B. Ephrussi. 1972. Gene dosage dependence of pigment synthesis in melanoma X fibroblast hybrids. Proc. Nat. Acad. Sci. U.S.A. **69**:330–334.
28. Ganschow, R. 1966. Glucuronidase gene expression in somatic hybrids. Science. **153**:84–85.
29. Gelehrter, T. D., J. R. Emanuel, and C. J. Spencer. 1972. Induction of tyrosine aminotransferase by dexamethasone, insulin, and serum. J. Biol. Chem. **247**:6197–6203.
30. Gordon, S., and Z. Cohn. 1970. Macrophage-melanocyte heterokaryons. I. Preparation and properties. J. Exp. Med. **131**:981–1003.
31. Gordon, S., and Z. Cohn. 1971. Macrophage-melanocyte heterokaryons. II. The activation of macrophage DNA synthesis. Studies with inhibitors of RNA synthesis. J. Exp. Med. **133**:321–338.
32. Gordon, S., and Z. Cohn. 1971. Macrophage-melanoma cell heterokaryons. III. The activation of macrophage DNA synthesis. Studies with inhibitors of protein synthesis and with synchronized melanoma cells. J. Exp. Med. **134**:935–946.
33. Gordon, S., and Z. Cohn. 1971. Macrophage-melanoma cell heterokaryons. IV. Unmasking the macrophage-specific membrane receptor. J. Exp. Med. **134**:947–962.
34. Green, H., B. Ephrussi, M. Yoshida, and D. Hamerman. 1966. Synthesis of collagen and hyaluronic acid by fibroblast hybrids. Proc. Nat. Acad. Sci. U.S.A. **55**:41–44.
35. Harris, H. 1970. Cell fusion. Clarendon Press, Oxford.
36. Harris, H., J. F. Watkins, C. E. Ford, and G. I. Schoefl. 1966. Artificial heterokaryons of animal cells from different species. J. Cell Sci. **1**:1–30.
37. Jacob, F., D. Perrin, C. Sanchez, and J. Monod. 1960. L'Opéron: groupe de gènes à expression coordonnée par un opérateur. C.R. Acad. Sci. Paris **250**:1727–1729.
38. Jacobson, C. O. 1968. Reactivation of DNA synthesis in mammalian neuron nuclei after fusion with cells of an undifferentiated fibroblast line. Exp. Cell Res. **53**:316–318.
39. Jami, J., C. Failly, and E. Ritz. 1973. Lack of expression of differentiation in teratoma-fibroblast somatic cell hybrids. Exp. Cell Res. **76**:191–199.
40. Kano, K., B. Knowles, H. Koprowski, and F. Milgrom. 1971. HL-A antigens on man-mouse hybrid cells. Fed. Proc. **30**:691.
41. Kao, F. T., and T. T. Puck. 1972. Genetics of somatic mammalian cells: demonstration of a human esterase activator gene linked to the adeB gene. Proc. Nat. Acad. Sci. U.S.A. **69**:3273–3277.
42. Klebe, R. J., T. Chen, and F. H. Ruddle. 1970. Mapping of a human genetic regulator element by somatic cell genetic analysis. Proc. Nat. Acad. Sci. U.S.A. **66**:1220–1227.
43. Klein, E., and F. Wiener. 1971. Loss of surface-bound immunoglobulin in mouse A9 and human lymphoblast hybrid cells. Exp. Cell Res. **67**:251.
44. Levisohn, S. R., and E. B. Thompson. 1971. Differentiated functions in HTC-L cell hybrids. In Vitro **6**:385.
45. Littlefield, J. W. 1969. Hybridization of hamster

cells with high and low folate reductase activity. Proc. Nat. Acad. Sci. U.S.A. **62**:88-95.
46. Migeon, B. R., S. W. Smith, and C. L. Leddy. 1969. The nature of thymidine kinase in the human-mouse hybrid cell. Biochem. Genet. **3**:583-590.
47. Minna, J. D., and D. E. Glazer. 1971. Expression of the acetylcholine-esterase genome in neuroblastoma X L cell hybrids. Fed. Proc. **30**:1085.
48. Minna, J., D. Glazer, and M. Nirenberg. 1972. Genetic dissection of neural properties using somatic cell hybrids. Nature N. Biol. (London) **235**:225-231.
49. Minna, J., P. Nelson, J. Peacock, D. Glazer, and M. Nirenberg. 1971. Genes for neuronal properties expressed in neuroblastoma X L cell hybrids. Proc. Nat. Acad. Sci. U.S.A. **68**:234-239.
50. Mohit, B., and K. Fan. 1971. Hybrid cell line from a cloned immunoglobulin-producing mouse myeloma and a nonproducing mouse lymphoma. Science **171**:75-77.
51. Moore, G. P. M., and N. R. Ringertz. 1973. Localization of DNA-dependent RNA polymerase activities in fixed human fibroblasts by autoradiography. Exp. Cell Res. **76**:223-228.
52. Parkman, R., A. Hagemeier, and E. Merler. 1971. Production of a human γ-globulin fragment by human thymocyte-mouse fibroblast hybrid cells. Fed. Proc. **30**:530.
53. Periman, R. 1970. IgG synthesis in hybrid cells from an antibody-producing mouse myeloma and an L cell substrain. Nature (London) **228**:1086-1087.
54. Peterson, J. A., and M. C. Weiss. 1972. Expression of differentiated functions in hepatoma cell hybrids: induction of mouse albumin synthesis in rat hepatoma-mouse fibroblast hybrids. Proc. Nat. Acad. Sci. U.S.A. **69**:571-575.
55. Ringertz, N. R., S. A. Carlsson, T. Ege, and L. Bolund. 1971. Detection of human and chick nuclear antigens in nuclei of chick erythrocytes during reactivation in heterokaryons with HeLa cells. Proc. Nat. Acad. Sci. U.S.A. **68**:3228-3232.
56. Rosenberg, M. 1972. Fusion of frog and tadpole erythrocytes. Nature (London) **239**:520-522.
57. Ryter, A., and P. Benda. 1972. Etude au microscope éléctronique d'hybrides de cellules gliales et de fibroblastes. Exp. Cell Res. **74**:407-416.
58. Schneider, J. A., and M. C. Weiss. 1971. Expression of differentiated functions in hepatoma cell hybrids. I. Tyrosine aminotransferase in hepatoma-fibroblast hybrids. Proc. Nat. Acad. Sci. U.S.A. **68**:127-131.
59. Silagi, S. 1967. Hybridization of a malignant melanoma cell line with L cells *in vitro*. Cancer Res. **27**:1953-1960.
60. Sonnenschein, B., U. I. Richardson, and A. H. Tashjian. 1971. Loss of growth hormone production following hybridization of a functional rat pituitary cell strain with a mouse fibroblast line. Exp. Cell Res. **69**:336-344.
61. Sonnenschein, C., A. Tashjian, Jr., and U. I. Richardson. 1968. Somatic cell hybridization: mouse-rat hybrid cell line involving a growth hormone-producing parent. Genetics **60**:227-228.
62. Spencer, R. A., T. S. Hauschka, D. B. Amos, and B. Ephrussi. 1964. Co-dominance of isoantigens in somatic hybrids of murine cells grown *in vitro*. J. Nat. Cancer Inst. **33**:893-903.
63. Thompson, E. B., and T. D. Gelehrter. 1971. Expression of tyrosine aminotransferase activity in somatic cell heterokaryons: evidence for negative control of enzyme expression. Proc. Nat. Acad. Sci. U.S.A. **68**:2589-2593.
64. Tomkins, G. M., T. D. Gelehrter, D. Granner, D. Martin, Jr., H. H. Samuels, and E. B. Thompson. 1969. Control of specific gene expression in higher organisms. Science **166**:1474-1480.
65. Weiss, M. C., and M. Chaplain. 1971. Expression of differentiated functions in hepatoma cell hybrids: reappearance of tyrosine aminotransferase inducibility after the loss of chromosomes. Proc. Nat. Acad. Sci. U.S.A. **68**:3026-3030.
66. Weiss, M. C., and B. Ephrussi. 1966. Studies of interspecific (rat X mouse) somatic hybrids. II. Lactate dehydrogenase and β-glucuronidase. Genetics **54**:1111-1122.
67. Zubay, G., D. A. Chambers, and L. C. Cheong. 1970. Cell-free studies on the regulation of the *lac* operon, p. 375-392. *In* J. R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory.
68. Zubay, G., D. Schwartz, and J. Beckwith. 1970. Mechanism of activation of catabolite-sensitive genes: a positive control system. Proc. Nat. Acad. Sci. U.S.A. **66**:104-110.